

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: A01N 43/04, A61K 31/70	A1	(11) International Publication Number: WO 95/31105 (43) International Publication Date: 23 November 1995 (23.11.95)
(21) International Application Number: PCT/US95/05908 (22) International Filing Date: 11 May 1995 (11.05.95) (30) Priority Data: 08/242,268 13 May 1994 (13.05.94) US (60) Parent Application or Grant (63) Related by Continuation US 08/242,268 (CIP) Filed on 13 May 1994 (13.05.94) (71) Applicant (for all designated States except US): THOMAS JER- FERSON UNIVERSITY [US/US]; 11th & Walnut Streets, Philadelphia, PA 19107 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MASTRANGELO, Michael, J. [US/US]; 764 Glen Road, Jenkintown, PA 19046 (US). LATTIME, Edmund, C. [US/US]; 600 Sayre Drive, Princeton, NJ 08540 (US). BERD, David [US/US]; 125 Heacock Lane, Wyncote, PA 19095 (US). EISEN- LOHR, Laurence, C. [US/US]; 110 Rockland Avenue, Merion, PA 19006 (US).	(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, Woodland Falls Corporate Park, Suite 201, 219 Lake Drive East, Cherry Hill, NJ 08002 (US). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	
(54) Title: A METHOD OF INDUCING AN IMMUNE RESPONSE USING VACCINIA VIRUS RECOMBINANTS (57) Abstract A method of inducing expression of immune active cytokines in tumors <i>in situ</i> is provided wherein a vaccinia virus vector capable of inducing expression of a selected cytokine is generated and injected into a tumor so that cells of the tumor express the selected cytokine. A method of enhancing immunity in a host by administration of a vaccinia virus vector is also provided. Methods of treating cancer by administration of these vaccinia virus vectors are also provided.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

- 1 -

**A METHOD OF INDUCING AN IMMUNE RESPONSE USING
VACCINIA VIRUS RECOMBINANTS**

Background of the Invention

Numerous attempts have been made to modulate a host's
5 immune system as a means for treating cancers. Such attempts
include: active immunotherapy using tumor or tumor antigen
containing vaccines or immune active lymphokines; adoptive
immunotherapy using a host's peripheral blood or tumor
infiltrating lymphocytes expanded in culture and reinjected;
10 passive immunotherapy by administration of monoclonal
antibodies; and localized immunotherapy using intralesional
administration of agents such as Bacillus Calmette-Guerin
(BCG). The most effective of these approaches has been
localized therapy with BCG for melanoma metastasis to the skin
15 and superficial bladder cancer. While the mechanism of action
of BCG is not completely understood, studies clearly show that
successful immunotherapy of this type is associated with
recruitment of T cells to the tumor.

Cytokines such as the interleukins are important
20 mediators in cell-mediated immune responses in a host. The
cell-mediated immune response ("local immune response") is
produced by thymus derived lymphocytes or T-cells. T-cells
detect the presence of invading pathogens through a recognition
system referred to as the T-cell antigen receptor. Upon
25 detection of an antigen, T-cells direct the release of multiple
T-cell lymphokines including, but not limited to, the
interleukin-2 family (IL-2). IL-2 is a T-cell growth factor
which promotes the production of many more T-cells sensitive to

- 2 -

the particular antigen. This production constitutes a clone of T-cells. The sensitized T-cells attach to cells containing the antigen. T-cells carry out a variety of regulatory and defense functions and play a central role in immunologic responses.

- 5 When stimulated to produce a cell-mediated immune response, some T-cells respond by acting as killer cells, killing the host's own cells when these have become infected with virus and possibly when they become cancerous and therefore foreign. Some T-cells respond by stimulating B cells while other T-cells
10 respond by suppressing immune responses.

- Examples of other interleukins which are mediators in cell-mediated immune responses include interferon- γ (IFN- γ), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-12
15 (IL-12). IFN- γ activates macrophages and enhances expression of immune-reactive antigens on tumor cells. GM-CSF activates macrophages and stimulates macrophage and dendrite cell recruitment and differentiation. IL-4 is a T cell derived helper lymphokine which participates in the regulation of
20 growth and differentiation of B and T cells. IL-5 is a T cell derived lymphokine which has its primary effects on the expansion of eosinphils. There is evidence which suggests that eosinphils, when recruited to a tumor site, may have direct anti-tumor effects. IL-12 is a heterodimeric lymphokine
25 initially purified from the conditioned medium of a human B lymphoblastoid cell line. Murine IL-12 has now been cloned and expressed. IL-12 stimulation has been shown to enhance antigen presentation and the cytolytic activity of natural killer cells.

- 30 The value of cytokine-based gene therapy was suggested in preclinical murine studies. Inoculation of mice with experimental tumors transfected with genes for tumor necrosis factor (Asher AL, et al., *J. Immunol.* 1991 146:3227), interleukin-2 (Fearon ER, et al., *Cell* 1990 60:397), and IL-4
35 (Golumbek PT, et al., *Science* 1991 254:713) resulted in growth and subsequent rejection of the injected tumor. In many cases the mice were shown to generate a systemic anti-tumor response.

- 3 -

IL-4 transfected tumors regressed and lead to the regression of admixed non-transfected tumors in mice (Tepper PI, et al., *Cell* 1989. 57:503). This immunotherapy was also effective in nu/nu mice demonstrating a non-T cell component which may contribute to localized therapy. IL-4 transfected RENCA cells have been shown to generate specific T cell immunity to the tumor, and result in elimination of pre-existing non-local tumor growth (Golumbek PT, et al., *Science* 1991 254:713).

Current approaches to this form of therapy involve the growth and stable gene modification of tumor cells to produce cytokines, their expansion *in vitro*, and reinjection into the host. While this type of therapy may be feasible in experimental systems, the lack of ability to grow the majority of tumors *in vitro*, the requirements for *in vitro* genetic modification of each patient's tumor, and the reinjection of viable tumor into the patient limit the clinical applicability of the approach.

It has now been found that expression of immune active cytokines in tumors can be induced *in situ* by administration of a vaccinia virus vector. These vaccinia virus vectors can be administered to animals suffering from cancer as a treatment. The vaccinia virus vectors of the present invention are also useful in enhancing immunity to parasites and other invading pathogens which alone fail to invoke an effective host immune response.

Summary of the Invention

An object of the present invention is to provide a method of inducing expression of immune active cytokines in tumors *in situ* which comprises generating a vaccinia virus vector capable of inducing expression of a selected cytokine and injecting the vaccinia virus vector into a tumor so that cells of the tumor express the selected cytokine.

Another object of the present invention is to provide a method of enhancing immunity in a host which comprises generating a vaccinia virus vector capable of inducing expression of a selected cytokine and injecting the vaccinia

- 4 -

virus vector into a host so that cells of the host express the selected cytokine.

A final object of the present invention is to provide a method of treating cancer which comprises administering to an animal suffering from cancer an amount of a vaccinia virus vector capable of inducing an immune response to the cancer in the animal.

Brief Description of the Figure

Figure 1 is a bar graph showing systemic immunity resulting from intravesical instillation of the vaccinia virus vector (VAC). Mice received intravesical instillation of VAC at 10, 100 and 1,000 pock forming units (pfu). Two weeks later, mice spleens were removed and tested for their ability to lyse VAC-infected MB-49 cells.

Detailed Description of the Invention

It has been clearly demonstrated in a number of studies that generation of effective T-cell specific immunity can result in the elimination of tumors. *In vitro* transduced cytokine and viral genes expressed by tumors have resulted in the elimination of transfected tumors and enhanced T cell mediated immunity to non-transduced tumors. Expression of immune accessory molecules such as B7.1 and B7.2 has also been demonstrated to enhance anti-tumor immunity. However, *in vitro* manipulations of tumors to express selected molecules has its limitations, particularly in the clinical setting. Genetic modification of tumors for cellular vaccines is dependent upon and limited by the ability to resect and to grow each patient's tumor *in vitro* and reinjection of viable, modified tumor.

A method has now been developed for *in vivo* gene delivery of a gene which expresses an immune active cytokine which obviates the need for *in vitro* manipulations of tumor cells thus enhancing the clinical applicability of this therapeutic approach. In the present invention, a method of inducing expression of immune active cytokines in tumors *in situ* is provided which comprises generating a vaccinia virus vector

- 5 -

capable of inducing expression of a selected cytokine and injecting the vaccinia virus vector into a tumor so that cells of the tumor produce the selected cytokine. By the term "inducing" or "induces" it is meant that the level of expression of the cytokine is measurable by methods well known in the art and that the level of expression of the cytokine results in an immune response. By the term "immune active cytokine" or "selected cytokine" it is meant to refer to any cytokine associated with an immune response leading to tumor destruction. Examples of such cytokines include, but are not limited to, interferon- γ (IFN- γ), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-12 (IL-12). The vaccinia virus vector may further comprise a gene for an immune accessory molecule such as B7.1 or B7.2. By "immune accessory molecule" it is meant a molecule which in conjunction with the immune active cytokine can make the tumor more immunogenic. Unlike *in vitro* methods of gene transfer, infection and transfection using recombinant vaccinia has been found to be a simple, rapid and highly efficient procedure. Vaccinia recombinants can efficiently deliver antigens to the class I presentation pathway and have been proposed as feasible vectors for expressing protective antigens for vaccine delivery. Moss B and Flexner C., "Vaccinia virus expression vector", *Ann. Rev. Immunol.* 1987 5:305-324. The potential utility of vaccinia recombinants for intravesical gene therapy aimed at enhancing the immunogenicity of bladder tumor cells was suggested by Lee SS, et al. *Proc. Am. Assoc. Cancer* March 1993 34:337. It has now been found that these viral vectors can be used in a method of stimulating the immune system by inducing expression of cytokines at a tumor site.

Vaccinia virus, a double stranded DNA poxvirus, has been well characterized since its successful use as a live vaccine to prevent smallpox. As a versatile eukaryotic expression vector, vaccinia virus can be genetically constructed to contain large fragments of foreign DNA (up to 25 kd) which have no effect on viral replication. Immunization with recombinant

- 6 -

vaccinia can induce protective responses to the foreign gene(s) expressed. In the present invention a vaccinia virus vector (VAC) capable of inducing expression of a selected gene is generated in accordance with methods well known in the art.

5 The vaccinia virus vector may further comprise genes encoding immune accessory molecules which in conjunction with the immune active cytokine can make the tumor more immunogenic.

In a preferred embodiment, a foreign gene of interest, preferably a gene for a selected cytokine, more preferably the
10 gene for IFN- γ , GM-CSF, IL-4, IL-5 or IL-12, is first placed behind a promoter, preferably a VAC promoter, in a plasmid that can be inserted into the VAC genome by homologous recombination. Other genes which may be incorporated into the vector include, but are not limited to, genes encoding immune
15 accessory molecules such as B7.1 and B7.2, or genes which inhibit IL-10 production. It has recently been found that both human melanoma and bladder cancer produce the immunosuppressive cytokine IL-10. Thus, inhibition of this cytokine is believed to enhance the immunogenicity of tumors. Inhibition of the
20 expression of IL-10 has been demonstrated through the use of antisense oligonucleotides complementary to the IL-10 DNA or mRNA in other cells. The ability to express an antisense oligonucleotide complementary to the IL-10 DNA or mRNA can be incorporated into vaccinia virus vectors of the present
25 invention to inhibit IL-10 production in tumor cells, thus enhancing the immunogenicity of these tumors.

Successful insertion of the selected gene in the plasmid is confirmed by exploiting the high transfectability of certain cell lines following vaccinia infection. After 30 minutes of
30 exposure to wild-type vaccinia at a multiplicity of 10:1, mouse L929 cells are transfected with a plasmid DNA-lipofectin (Gibco/BRL, Bethesda, MD) mixture. Within hours of transfection, abundant amounts of gene product can be observed, with a majority of the cells expressing the protein. The
35 generation of the desired gene can be detected using standard immunodetection techniques such as immunoprecipitation of metabolically-labeled proteins or western blot of cell-

- 7 -

extracts. Further, supernatants from the infected/transfected cells are tested for biological activities associated with the various cytokines or other proteins. After confirmation that the gene of interest has been correctly inserted and encodes a biologically active protein, the plasmid is recombined in the VAC genome. The plasmids are designed such that the gene of interest is inserted between the up- and downstream halves of the VAC thymidine kinase gene. Following infection of CV-1 monkey kidney cells with non-recombinant virus, the plasmid is delivered using calcium phosphate precipitation. In a portion of the cells, the plasmid recombines into the vaccinia genome, disrupting the thymidine kinase gene. The resulting recombinants are then selected from wild-type by growth in thymidine kinase negative 143B human osteosarcoma cells in the presence of bromodeoxyuridine. It is preferred that the Wyeth strain of vaccinia, available from the Centers for Disease Control in Atlanta, GA (CDC) be used as this strain was used for small pox vaccinations in the United States. However, attenuated strains of vaccinia may also be used if immunogenicity following attenuation is not significantly compromised.

Susceptibility of cells to the vaccinia virus was demonstrated in *in vitro* experiments in both murine and human tumor cells. Both type of cells were infected/transfected by vaccinia recombinants. Significant infection/transfection of established tumors in mice was also observed following intravesical administration. Systemic immunity to vaccinia did not inhibit tumor transfection by intravesically instilled vaccinia recombinants.

The safety and maintained function of the viral gene over repeated administrations have also been demonstrated in humans. Five patients with dermal, subcutaneous and/or lymph node metastases from cutaneous melanoma were vaccinated with wild-type vaccinia virus and, four days later, began intratumoral injections of the same vaccine. Escalating doses of up to 10^7 pfu were safely administered repeatedly with only local and mild systemic reactions. Four of the patients developed anti-

- 8 -

vaccinia virus antibody titers $\geq 1/3200$. With rising antibody titers, local and systemic reactions decreased. One patient with a large exophytic lesion experienced dramatic tumor regression with multiple injections of 10^7 pfu of virus.

5 Sequential biopsies of this lesion over a two month period demonstrated repeated infection over successful production of viral gene protein (E3L) despite anti-viral antibody titers as high as $1/12,800$. This time interval is adequate to allow generation of anti-tumor immunity. It is believed that a

10 vector comprising a cytokine gene would function similarly and mediate an immunoadjuvant effect.

The vaccinia virus vectors of the present invention can also be used to enhance immunity in a host. In the present invention methods of enhancing immunity in a host are provided

15 which comprise generating a vaccinia virus vector capable of inducing expression of a selected cytokine and injecting the vaccinia virus vector into a host so that cells of the host express the selected cytokine. By "host" it is meant to include, but is not limited to, mammals, fish, amphibians,

20 reptiles, birds, marsupials, and most preferably, humans. This method is also useful in enhancing a host's immune response to parasites and other invading pathogens which alone may not invoke an immune response.

In addition, the vaccinia virus vectors of the present

25 invention can be used to mediate cytokine gene transfer into tumors with resultant production of soluble product. For example, a recombinant vaccinia virus containing the murine GM-CSF gene under the control of the early/late P7.5 vaccinia promoter (VV-GM) was constructed. VV-GM infected murine

30 melanoma (B16.F10) and bladder (MB49) tumors were shown to produce high levels of biologically active cytokine as determined by propagation of bone marrow CFU-GM and by ELISA assay. Significant levels of GM-CSF were found in the supernatant as soon as 6 hours following infection. This

35 increased cytokine secretion of the tumor cells can lead to tumor specific immunity and therapeutic anti-tumor effects.

Accordingly, the vectors and methods of the present

- 9 -

invention are useful in the treatment of cancer. Methods of treating cancer are provided comprising administering to an animal suffering from cancer an amount of a vaccinia virus vector capable of inducing an immune response to the cancer in the animal. In a preferred embodiment, the vaccinia virus vector used comprises at least one gene for expression of a cytokine, preferably the gene for IFN- γ , GM-CSF, IL-4, IL-5 or IL-12. In this treatment, the vaccinia virus vector is placed in contact with the tumor *in situ* either by intravesical administration or by direct injection into the tumor. Therefore, this method is especially useful in treating cancers such as bladder cancer, head cancer, neck cancer, melanoma, and other cancers which grow as accessible masses and are amenable to these routes of administration.

The susceptibility of human prostatic carcinoma cells to vaccinia was also examined utilizing a recombinant vector encoding the human influenza hemagglutinin antigen HA. In vitro exposure of the prostatic cell lines LNCAP and PC3 to the virus followed by immunohistochemical staining of the encoded HA protein demonstrated a high efficiency in tumor infection/transfection. Thus, the vaccinia virus vectors of the present invention can also be used in the localized therapy of prostate cancer.

The vaccinia virus vectors of the present invention are administered in a vaccine formulation comprising an effective concentration of vaccinia virus vector and a pharmaceutically acceptable carrier. By "effective concentration" it is meant an amount of vaccinia virus vector which when administered to a tumor results in measurable expression of the selected cytokine and an enhanced immune response. Such amounts can be routinely determined by one of skill in the art in accordance with this disclosure. Pharmaceutically acceptable carriers include, but are not limited to saline solutions and buffered solutions. Suitable pharmaceutically acceptable carriers are well known in the art and are described for example in Gennaro, Alfonso, Ed., *Remington's Pharmaceutical Sciences*, 18th Edition 1990, Mack Publishing Co., Easton, PA, a standard reference

- 10 -

text in this field. Pharmaceutical carriers may be selected in accordance with the intended route of administration and the standard pharmaceutical practice. The vaccine formulation may further comprise an adjuvant. Adjuvants are substances which
5 are added to therapeutic or prophylactic agents, for example vaccines or antigens used for immunization, to stimulate the immune response. Use of adjuvants in vaccines to enhance an immune response is well known in the art.

The present invention is further illustrated by the
10 following nonlimiting examples.

EXAMPLES

Example 1: Recombinant vaccinia virus

Recombinant vaccinia viruses H1-VAC and NP-VAC expressing the hemagglutinin (H1) and nucleoprotein (NP) genes derived
15 from influenza virus A/PR8/34 were used. Expression of both influenza polypeptides is under the control of the early/late 7.5 K promoter. Viral stocks quantitated in pfu were maintained in BSS/BSA at -70°C until use.

Example 2: Cell lines

20 The transitional cell carcinoma (TCC) cell lines MB-49 of C57BI origin, MBT-2 of C3H origin and the human T24 bladder carcinoma and H1 human melanoma were used.

Example 3: Antibodies, reagents and staining

Supernatants from hybridoma cell lines specific for the
25 influenza A hemagglutinin (H28-E23) and nucleoprotein antigens (HB65) were used to stain cells and tissues. The virus infected bladder tumor cells and bladder urothelium sections were fixed with cold acetone and blocked with 0.1% fetal calf serum. HA and NP were detected with primary mouse antibody and
30 biotin labeled antimouse IgG as the second antibody plus avidin-horseradish peroxidase (HRP) and 3.3 DAB substrate (Sigma, St. Louis, MO) or avidin-biotin-complex method (ABC-AP) plus alkaline phosphatase with Fast red substrate (Vector Laboratories, Inc., Burlingame, CA). Tissue sections were

- 11 -

counterstained with hematoxylin. In addition, hematoxylin-eosin (H&E) stained sections were prepared.

Example 4: In vitro assessment of viral infection and transfection

5 Cells (2×10^6) from each cell line described in Example 2 were plated into a 24 well flat bottom plate (Fisher, Pittsburgh, PA). Plates were incubated overnight, washed with phosphate buffered saline (PBS) and infected with H1-VAC or NP-VAC (10 pfu/cell) in BSS/BSA by incubating at 37°C, 9% CO₂ for
10 1 hour with rocking every 15 minutes. Virus was aspirated, media was added and the plate were incubated for another 4 hours. The cells were fixed with 1:1 acetone:methanol for 1 minute and washed with PBS before immunohistochemical staining. Uninfected and recombinant virus infected L929 fibroblasts,
15 which are known to be susceptible to vaccinia virus infection, were used as a negative and positive control, respectively.

 The murine MBT-2 and MB-49 TCC cells were infected in vitro with H1-VAC. When compared to uninfected tumor cells, immunohistochemical staining with specific antibodies showed
20 positive expression for encoded HA or NP antigens indicated by the cytoplasmic staining of virus infected TCC cells. In addition, the human bladder tumor cell line T24 and a human melanoma line were similarly infected in vitro.

25 **Example 5: In vivo assessment of virus infection and transfection**

 Female mice, 4-6 weeks of age, were purchased from the Jackson Laboratory, Bar Harbor, ME. The mice were intravesically instilled with recombinant vaccinia virus. Mice were anesthetized, catheterized via the urethra, then
30 cauterized with a cautery wire (Birtcher Hyfricator, El Monte, CA) by applying a single 1 second pulse at 1 watt. After removal of the cautery wire, the bladders were instilled with 10^4 MB49 cells to establish intravesical growth of a tumor or either 10, 100 or 1,000 pfu of vaccinia virus recombinants in
35 PBS. At 8 and 22 hours following instillation, mice were sacrificed and bladders were removed and frozen in OCT media

- 12 -

(Fisher) in liquid nitrogen. Bladder samples were stored at -70°C until sectioned.

Mice, pre-immunized intraperitoneally with wild-type WR vaccinia (10^7 pfu), were implanted intravesically with MB-49 tumor cells. Two weeks following tumor development, a single intravesical instillation of NP-VAC (2×10^6 pfu, shown not to have systemic toxicity in preimmune mice) was given. At 8 and 22 hours post-instillation, bladders were removed, sectioned and stained. *In vivo* expression of encoded NP was demonstrated at 22 hours after instillation. Similar results were seen at the 8 hour time point.

Example 6: Cytotoxic T Lymphocyte analysis

Cytotoxic T Lymphocyte (CTL) responses to intravesical infection by vaccinia recombinants were determined by a 4 hour ^{51}Cr assay. Spleens of virus infected mice were isolated at 2 weeks post-intravesical instillation, restimulated *in vitro* with live virus infected syngeneic spleen stimulators (3:1) and cultured for 7 days at 37°C, 5% CO_2 . The responder cells were assayed for cytotoxicity on ^{51}Cr labeled vaccinia virus infected MB49 tumor targets at effector to target ratios indicated. Percent specific lysis was calculated as follows: $(\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{total release} - \text{spontaneous release}) \times 100$. Spleens of intravesically infected C57BL/6 mice were tested for antigen specific killing of vaccinia virus infected MB-49 bladder tumor target cells in 4 hour chromium release assays. No virus-induced target lysis was seen in the 4 hour assay and virus-specific CTL did not lyse uninfected targets. As shown in Figure 1, concentrations as low as 10^5 pfu intravesically were sufficient to induce a systemic anti-vaccinia CTL response. When the dose of intravesical vaccinia was titrated, concentrations of greater than 10^5 pfu per mouse were lethal to nonimmunized mice, which died within 5-6 days post-instillation. In contrast, mice receiving a single intravesical concentration less than 10^5 pfu appeared normal and survived greater than 2 weeks post-instillation. Mice made preimmune with an intraperitoneal

- 13 -

injection of wild-type WR vaccinia virus (10^7 pfu) demonstrated no morbidity at intravesical concentrations as high as 2×10^6 pfu of vaccinia recombinants per mouse.

C57BL/6 female mice were given a single intravesical
5 instillation with vaccinia recombinant H1-VAC or NP-VAC (10^4
pfu) to confirm infection of the urothelium. The mice were
sacrificed, post instillation, and their bladders were
recovered for sectioning and staining. Analysis of the bladder
wall by routine pathology procedures using H & E stained slides
10 demonstrated that urothelial cells lining the bladder lumen
were virus infected as indicated by characteristic morphologic
changes including cell enlargement, nuclear and cytoplasmic
vacuolization, as well as atypical chromatin pattern.

15 **Example 7: Human study using intratumoral vaccinia injections
as a vector for gene transfer**

Patients in this study each had histologically
documented, surgically incurable melanoma with at least one
dermal, subcutaneous or lymph node metastasis which was
evaluable for local response and accessible for injection.
20 Eligible patients were fully ambulatory with or without minor
tumor related symptoms, had a life expectancy of six or more
months and were at least four weeks since surgery (requiring
general anesthesia) and eight weeks since chemotherapy or
radiation therapy. All patients were immunocompetent as
25 demonstrated by one or more positive cutaneous delayed-type
hypersensitivity reactions to recall microbial antigens or to
dinitrofluorobenzene after sensitization.

Patients were administered Dryvax (Wyeth-Ayerst
Laboratories, Philadelphia, PA) supplied by the Center for
30 Disease Control (Atlanta, GA) in a lyophilized state. When
reconstituted as directed, the resultant product contains 25
million pfu in a volume of 0.25 ml.

Each patient was vaccinated, using a standard
multipuncture method with a bifurcated vaccination needle, on
35 the skin of the deltoid area which in all cases was a tumor
free extremity with intact regional lymph nodes. The

- 14 -

vaccination site was evaluated visually on day 4 to confirm that a major local reaction (erythematous papule with vesiculation and pustule formation) was in progress. Tumor treatment commenced on day 4. Dermal, subcutaneous and/or lymph node metastases were infiltrated with wild-type vaccinia virus by intralesional injection using a 25 gauge needle (volume of injection ranged from 0.05 to 0.1 ml). Treatment was repeated approximately twice weekly.

Regression of injected and uninjected lesions was judged by visual inspection and/or ultrasonography. Ultrasonography was performed using a 10.0 MHz linear probe (Advanced Technology Laboratories, Inc., Bothel, WA) with direct contact scanning of the surface of the mass as well as scanning with a stand-off pad (Parker Laboratories, Inc., Orange, NJ). All masses were imaged in the sagittal and axial planes. Tumor location, depth of penetration and sonographic textural appearance were determined. Tumors were measured in millimeters (mm), with the sagittal (S) and anteroposterior (AP) dimensions taken from the sagittal image with the greatest dimension. The tumor width (W) was obtained from the transverse plane. Lesional response were categorized as complete (no clinically evident residual tumor), partial ($\geq 50\%$ reduction in tumor volume) or none (all others).

Punch biopsies were also performed using conventional sterile dermatologic techniques. One half of the material was fixed in formalin, paraffin embedded and sections stained with hematoxylin and eosin for routine histology. The remaining tissue was halved again for transmission electron microscopy (EM) and immunohistochemical analysis. Tissue was either fixed in 2% glutaraldehyde or embedded in OCT (Fisher Scientific, Pittsburgh, PA) and snap frozen using liquid nitrogen. Frozen tissue was subsequently sectioned at 5 microns thickness with a cryostat, fixed in cold acetone, blocked with PBS with 5% fetal calf serum (FCS) and stained with the antibody TW2.3 which is specific for an early gene product of vaccinia virus replication (E3L). As E3L is a non-structural viral protein, positive antibody staining is indicative of active infection.

- 15 -

To measure serum titers for anti-vaccinia virus antibody, ninety-six well plates were coated with a 10 µg/ml protein extract obtained from cultures of human melanoma cell lines infected for 6 hours with the Wyeth strain of vaccinia virus.

5 Following blocking with PBS plus FCS, dilution series of patient sera pre- and post-immunization were added to the wells, incubated for two hours and the plates washed. Serum anti-vaccinia virus antibodies were visualized using a peroxidase labeled anti-human IgG heavy and light chain second

10 reagent and orthophenyldiamine substrate. Titers were read as the reciprocal serum dilution yielding 50% maximum absorbance in the assay.

Example 8: Intralesional infection of human melanoma cells by vaccinia virus

15 One patient, a sixty-five year old white female, was first diagnosed with a primary melanoma (1 mm, level 4) of the right calf with satellite lesions in 1983. The primary lesion was excised and the dermal satellites successfully treated with intratumoral BCG. The patient did well until 1992 when two

20 dermal/sc lesions appeared on the calf and failed to respond to intratumoral BCG, systemic R24 or chemotherapy. Vaccinia treatment was initiated with a standard immunization (250,000 pfu topically, 15 punctures). On day 4 of treatment, when it was determined that a take was clearly in progress,

25 intralesional vaccinia was commenced. A single metastatic lesion was injected 19 times over 88 elapsed days with a total of 14×10^7 pfu (Wyeth). Several biopsies showed progressively intense infiltration of the tumor with lymphocytes and tumor regression. EM and immunohistological staining for vaccinia

30 gene products showed successful viral infection of tumor cells in the presence of substantial anti-vaccinia antibody titers.

Example 9: Clinical Trials

Patients are administered small pox vaccine (Dryvax, Wyeth Laboratories, by scarification. Immunity to the vaccine

35 is demonstrated by a major reaction characterized by pustule formation at the vaccination site and the detection of

- 16 -

circulating anti-vaccinia antibody. Patients exhibiting both response are eligible for localized treatment with the cytokine producing vaccinia vector.

Patients are treated with increasing doses of the
5 vaccinia over a several week period by local (intratumoral or topical such as intravesical) administration. In the case of melanoma, head and neck, and other tumors which grow as accessible solid masses at the primary and or metastatic sites, the vaccinia is injected into the tumor using a syringe and
10 needle. In the case of bladder cancer, the vaccinia is instilled onto the bladder (intravesically) using a catheter.

Patients are observed at frequent intervals for signs of toxicity, and tumor response is gauged by measuring the injected and non-injected tumor masses for signs of shrinkage
15 by direct visualization or radiologic (ultrasound, X-ray, MRI, etc.) methods.

Evidence of systemic antitumor immunity will be determined using *in vitro* techniques which measure the direct interaction of lymphocytes and tumor cells. Measurements of
20 antitumor immunity are readily accomplished by persons with skill in this field.

- 17 -

What is claimed is:

1. A method of inducing expression of immune active cytokines in tumors *in situ* comprising:
 - a) generating a vaccinia virus vector capable of
 - 5 inducing expression of a selected cytokine; and
 - b) injecting the vaccinia virus vector into a tumor so that cells of the tumor express the selected cytokine.
2. The method of claim 1 wherein the vaccinia virus vector is capable of inducing expression of interferon- γ ,
10 granulocyte-macrophage colony stimulating factor, interleukin-4, interleukin-5 or interleukin-12.
3. A method of enhancing immunity in a host comprising:
 - a) generating a vaccinia virus vector capable of
 - inducing expression of a selected cytokine; and
 - 15 b) injecting the vaccinia virus vector into a host so that cells of the host express the selected cytokine.
4. The method of claim 3 wherein the vaccinia virus vector further comprises a gene for an immune accessory molecule.
- 20 5. The method of claim 3 wherein the vaccinia virus vector further comprises a sequence encoding an oligonucleotide which inhibits IL-10 production.
6. A method of treating cancer comprising administering to an animal suffering from cancer an amount of a vaccinia
25 virus vector capable of inducing an immune response to the cancer in the animal.
7. The method of claim 6 wherein the vaccinia virus vector comprises at least one gene for expression of a cytokine.

- 18 -

8. The method of claim 7 wherein the vaccinia virus vector comprises at least one gene for expression of interferon- γ , granulocyte-macrophage colony stimulating factor, interleukin-4, interleukin-5 or interleukin-12.

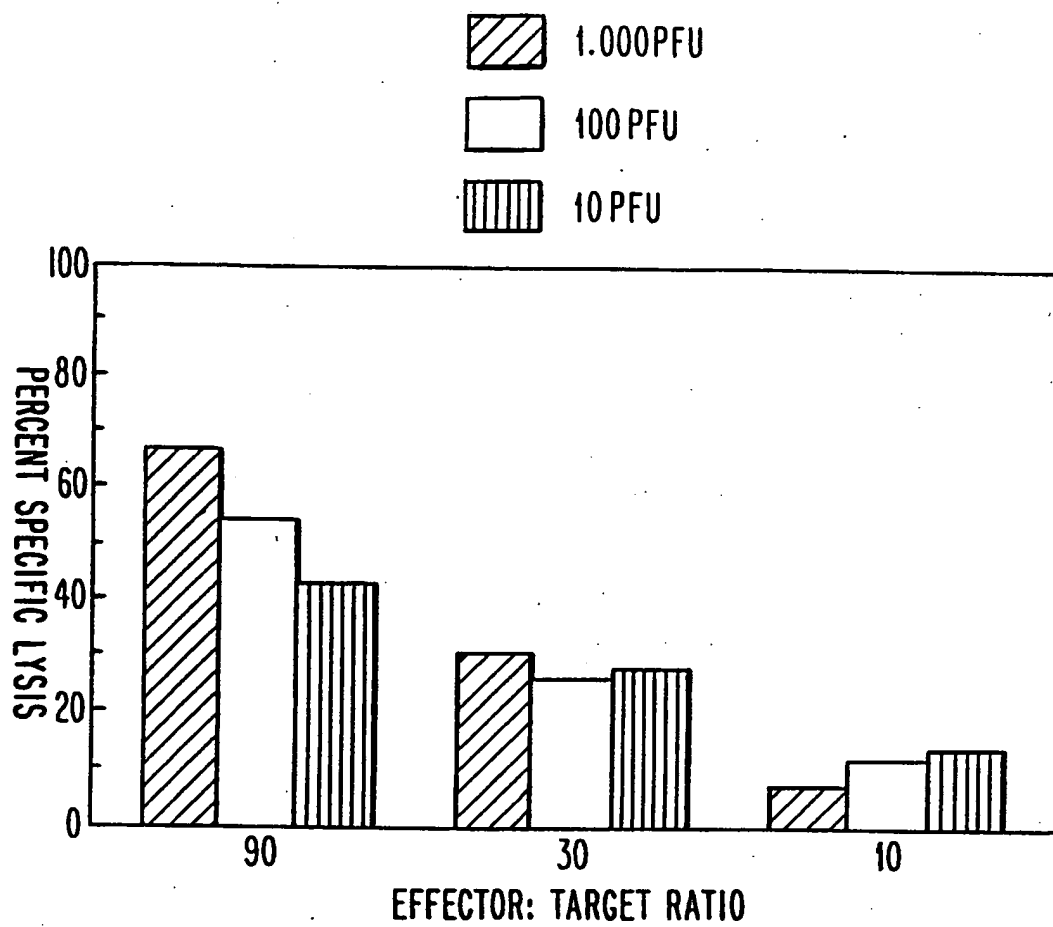
5 9. The method of claim 6 wherein the cancer to be treated comprises bladder cancer, head cancer, neck cancer, melanoma, or other cancers which grow as accessible masses.

10. The method of claim 6 wherein the cancer to be treated comprises prostate cancer.

10 11. The method of claim 7 wherein the vaccinia virus vector further comprises a gene for an immune accessory molecule.

12. The method of claim 7 wherein the vaccinia virus vector further comprises a sequence encoding an oligonucleotide
15 which inhibits IL-10 production.

1/1

***Fig. 1***

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05908

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04; A61K 31/70

US CL :514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/89; 428/88; 530/395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Lancet, Volume 339, issued 21 March 1992, A. GUTIERREZ ET AL., "Gene Therapy for Cancer", pages 715-721. See entire document, especially pages 716-717.	1-12
Y	Cancer(supp), Volume 70, Number 6, issued 15 September 1992, T. FRIEDMANN, "Gene Therapy of Cancer Through Restoration of Tumor Suppressor Functions", See entire document, especially page 1815.	1-12
Y	Proceedings of the American Association of Cancer Research, Volume 34, issued March 1993, LEE, "Intravesicular Gene Therapy: Vaccinia Virus Recombinants Transfect Murine Bladder Tumors and Urothelium", page 337, abstract number 2005. See entire abstract number.	1-3, 6-9

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
A	document defining the general state of the art which is not considered to be of particular relevance		
B	earlier document published on or after the international filing date	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O	document referring to an oral disclosure, use, exhibition or other means		
P	document published prior to the international filing date but later than the priority date claimed	A	document member of the same patent family

Date of the actual completion of the international search

09 AUGUST 1995

Date of mailing of the international search report

05.09.95

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Andrew K. Milne

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05908

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 57, issued 1989, TEPPER ET AL, "Murine Interleukin-4 Displays Potent Anti-Tumor Activity In Vivo", pages 503-512. See entire document.	1-3, 6-9
Y	Cancer Research, Volume 53, issued September 1993, VILE ET AL, "Use of Tissue-Specific Expression of the Herpes Simplex Virus Thymidine Kinase Gene to Inhibit Growth of Established Murine Melanomas following Direct Intratumoral Injection of DNA", pages 3860-3864. See entire document, especially page 3860.	1-3, 6-9
Y	Cancer Research, Volume 53, issued March 1993, VILE ET AL, "In vitro and In Vivo Targeting of Gene Expression to Melanoma Cells", pages 962-967. See entire document.	1-3, 6-9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05908

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS MEDLINE EMBASE JAPIO BIOSIS CAPLUS USPAT

search terms: vaccinia virus vector, intratumoral, direct injection, cancer, cytokine